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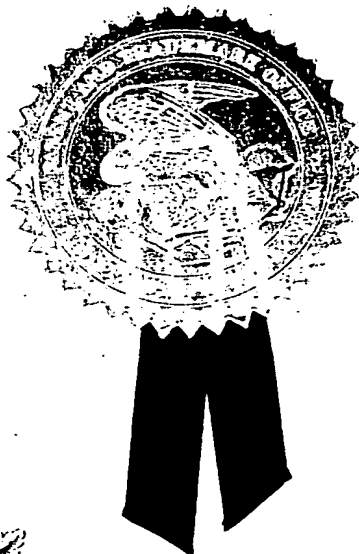
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APPLICATION NUMBER: 60/024,279

FILING DATE: August 21, 1996

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PATENT APPLICATION SERIAL NO. 60/024279

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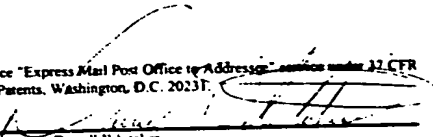
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I hereby certify that this paper or fee is being deposited with the U.S. Postal Service "Express Mail Post Office to Address" station under 37 C.F.R. 1.10 on the date indicated above and is addressed to Assistant Commissioner for Patents, Washington, D.C. 20231.

By:   
 Name: Darrell Whitaker

## REQUEST FOR PROVISIONAL APPLICATION UNDER 37 C.F.R. § 1.53(b)(2)

Assistant Commissioner for Patents  
 Washington, DC 20231

Dear Sir:

This is a request for filing a Provisional application for patent under 37 CFR § 1.53(b)(2) entitled IMPROVED NUCLEIC ACIDS ENCODING A CHIMERIC GLYCOSYLTRANSFERASE by the following inventor(s):

Full Name Of Inventor	Family Name MCKENZIE	First Given Name Ian Farquhar	Second Given Name Campbell
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Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
Post Office Address	Post Office Address	City	State & Zip Code/Country

1. ☒ Enclosed is the Provisional application for patent as follows: 28 pages of specification, and 7 sheets of drawings.
2. ☐ A Verified Statement that this filing is by a small entity (37 CFR 1.9, 1.27, 1.28) is attached.
3. ☒ Payment of Provisional filing fee under 37 C.F.R. § 1.16(k):
  - ☐ Attached is a check in the amount of \$ 150.00.
  - ☐ Please charge Deposit Account No. 13-2725.
  - ☐ PAYMENT OF THE FILING FEE IS BEING DEFERRED.

4. ☒ The Commissioner is hereby authorized to charge any additional fees as set forth in 37 CFR §§ 1.16 to 1.18 which may be required by this paper or credit any overpayment to Account No. 13-2725.
5. ☐ Enclosed is an Assignment of the invention to \_\_\_\_\_, Recordation Form Cover Sheet and a check for \$ \_\_\_\_\_ to cover the Recordation Fee.
6. ☐ Also Enclosed:
7. ☐ The invention was made by the following agency of the United States Government or under a contract with the following agency of the United States Government:
8. ☒ Address all future communications to the Attention of Denise M. Kettelberger, Ph.D. (may only be completed by attorney or agent of record) at the address below.
9. ☒ A return postcard is enclosed.

Respectfully submitted,  
Ian Farquhar Campbell McKenzie, et al.  
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Dated: August 21, 1996

By: Denise M. Kettelberger

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DMK:mel



AUSTRALIA  
Patents Act 1990

**PROVISIONAL SPECIFICATION**

**Applicant(s):**

**THE AUSTIN RESEARCH INSTITUTE**

**Invention Title:**

**IMPROVED NUCLEIC ACIDS ENCODING A  
CHIMERIC GLYCOSYLTRANSFERASE**

**The invention is described in the following statement:**



150:714 APPROV.

- 2 -

68/024279

IMPROVED NUCLEIC ACIDS ENCODING A CHIMERIC  
GLYCOSYLTRANSFERASE

Field of the Invention

5 The present invention relates to nucleic acids  
which encode glycosyltransferase and are useful in  
producing cells and organs from one species which may be  
used for transplantation into a recipient of another  
species. Specifically the invention concerns production of  
nucleic acids which, when present in cells of a  
10 transplanted organ result in reduced levels of antibody  
recognition of the transplanted organ.

Background of the Invention

The transplantation of organs is now possible due  
to major advances in surgical and other techniques.  
15 However, availability of suitable human organs for  
transplantation is a significant problem. Demand outstrips  
supply. This has caused researchers to investigate the  
possibility of using non-human organs for transplantation.

Xenotransplantation is the transplantation of  
20 organs from one species to a recipient of a different  
species. Rejection of the transplant in such cases is a  
particular problem, especially where the donor species is  
more distantly related, such as donor organs from pigs and  
sheep to human recipients. Vascular organs present a  
25 special difficulty because of hyperacute rejection (HAR).

HAR occurs when the recipient's complement is  
initiated by binding of antibodies to donor endothelial  
cells.

Previous attempts to prevent HAR have focused on  
30 two strategies : modifying the immune system of the host  
Systemic complement inhibition (1,2) and antibody depletion  
(3,4). Both strategies have been shown to temporally  
prolong xenograft survival. However, these methodologies  
are therapeutically unattractive in that they are



clinically impractical and would require chronic immunosuppressive treatments. Therefore, recent efforts to inhibit HAR have focused on genetically modifying the donor xenograft. One such strategy has been to achieve high-level expression of species-restricted human complement inhibitory proteins in vascularized pig organs via transgenic engineering (5-7). This strategy has proven to be useful in that it has resulted in the prolonged survival of porcine tissues following antibody and serum challenge (5,6). Although increased survival of the transgenic tissues was observed, long-term graft survival was not achieved (6). As observed in these experiments and also with systemic complement depletion, organ failure appears to be related to an acute antibody-dependent vasculitis (1,5).

In addition to strategies aimed at blocking complement activation on the vascular endothelial cell surface of the xenograft, recent attention has focused on identification of the predominant xenogeneic epitope recognised by high-titre human natural antibodies. It is now accepted that the terminal galactosyl residue, Gal $\alpha$ -1,3-Gal, is the dominant xenogeneic epitope (8-15). This epitope is absent in Old World primates and humans because the  $\alpha$ -1,3-galactosyltransferase (gal-transferase or GT) is non-functional in these species. DNA sequence comparison of the human gene to  $\alpha$ 1,3-galactosyltransferase genes from the mouse (16,17), ox (18), and pig (12), revealed that the human gene contained two frameshift mutations, resulting in a non-functional pseudogene (20,21). Consequently, humans and Old World primates have pre-existing high-titre antibodies directed at this Gal $\alpha$ -1,3-Gal moiety as the dominant xenogeneic epitope.

One strategy developed stably reduced the expression of the predominant Gal $\alpha$ -1,3-Gal epitope. This strategy took advantage of an intracellular competition between the gal-transference and the  $\alpha$ -1,2-

5 fucosyltransferase (H-transferase) for a common acceptor  
substrate. The gal-transferase catalyses the transfer of a  
terminal galactose moiety to an N-acetyl lactosamine  
acceptor substrate resulting in the formation of the  
terminal Gal $\alpha$ -1,3-Gal epitope. Conversely, H-transferase  
catalyses the transfer of a fucosyl residue to the N-acetyl  
lactosamine acceptor substrate and generates a fucosylated  
N-acetyl lactosamine (H-antigen, that is, the O blood group  
antigen), a glycosidic structure that is universally  
10 tolerated. Although it was reported that transfected cells  
expressing human H-transferase resulted in high level  
expression of the non-antigenic H-epitope and significantly  
reduced the expression of the Gal $\alpha$ -1,3-Gal xenoepitope,  
there are still significant levels of Gal $\alpha$ -1,3-Gal epitope  
15 present on such cells.

#### Summary of the Invention

20 In view of the foregoing, it is an object of the  
present invention to further reduce levels of undesirable  
epitopes in cells, tissues and organs which may be used in  
transplantation.

In work leading up to the invention the inventors  
surprisingly discovered that the activity of H transferase  
may be further increased by making a nucleic acid which  
encodes a H transferase catalytic domain but is anchored in  
25 the cell at a location where it is better able to compete  
for substrate with gal transferase. Although work by the  
inventors focused on a chimeric H transferase other  
glycosyltransferase enzymes may also be produced in  
accordance with the invention.

30 Accordingly, in a first aspect the invention  
provides a nucleic acid encoding a chimeric enzyme, wherein  
said chimeric enzyme comprises a catalytic domain of a  
first glycosyltransferase and a localisation signal of a  
second glycosyltransferase whereby when said nucleic acid  
35 is expressed in a cell said chimeric enzyme is located in

an area of the cell where it is able to compete for substrate with a second glycosyltransferase resulting in reduced levels of product from said second glycosyltransferase.

5           The term "nucleic acid" refers to any nucleic acid comprising natural or synthetic purines and pyrimidines. The nucleic acid may be DNA or RNA, single or double stranded or covalently closed circular.

10           Preferably the nucleic acid is in an isolated form; that is the nucleic acid is, at least, partly purified from other nucleic acids or proteins.

15           Preferably the nucleic acid comprises the correct sequences for expression, more preferably for expression in a eukaryotic cell. The nucleic acid may be present on any suitable eukaryotic expression vector such as pCDNA (Invitrogen). The nucleic acid may also be present on other vehicles whether suitable for eukaryotes or not, such as plasmids, phages and the like.

20           The term "catalytic domain" of the chimeric enzyme refers to the amino acid sequences necessary for the enzyme to function catalytically. This comprises one or more contiguous or non-contiguous amino acid sequences. Other non-catalytically active portions also may be included in the chimeric enzyme.

25           The term "glycosyltransferase" refers to a polypeptide with an ability to move carbohydrates from one molecule to another.

30           Preferably the catalytic domain of the first glycosyltransferase is derived from H transferase, secretor sialtransferase, a galactosyl sulphating enzyme or a phosphorylating enzyme.

35           The term "derived" from means that the catalytic domain is based on, or is similar to that of a native enzyme. The nucleic acid sequence encoding the catalytic domain is not necessarily directly derived from the native gene. The nucleic acid sequence may be made by PCR,

constructed de novo or cloned.

The nucleic acid sequence encoding the catalytic domain may be derived from, or similar to a glycosyltransferase from any species. Preferably said species is a mammalian species such as human or other primate species, including Old World monkeys, or other mammals such as ungulates (ie. pigs, sheep, goats, cows, horses, deer, camel) or dogs, mice, rats and rabbits. The term "similar to" means that the nucleic acid is at least partly homologous to the glycosyltransferase genes described above. The term also extends to fragments of and mutants, variants and derivatives of the catalytic domain whether naturally occurring or man made.

The term "localisation signal" refers to the amino acid sequence of a glycosyltransferase which is responsible for anchoring it in location within the cell. Generally localisation signals comprise amino terminal "tails" of the enzyme. The localisation signals are derived from a second glycosyltransferase, the activity of which it is desired to minimise. The localisation of a catalytic domain of a first enzyme in the same area as the second glycosyltransferase means that the substrate reaching that area is likely to be acted on by the catalytic domain of the first enzyme enabling amount of substrate catalysed by the second enzyme to be reduced.

Preferably the localisation signal is derived from a glycosyltransferase which produces glycosylation patterns which are recognised as foreign by a transplant recipient. More preferably the localisation signal is derived from  $\alpha$  (1,3) galactosyltransferase. The effect of this is to down regulate the level of Gal  $\alpha$ (1,3) Gal produced in a cell when the nucleic acid is expressed by the cell.

The nucleic acid sequence encoding the localisation signal may be derived from any species such as those described above. Preferably it is derived from the

same species as the cell which the nucleic acid is intended to transform i.e., if pig cells are to be transformed, preferably the localization signal is derived from pig.

5 More preferably the nucleic acid comprises a nucleic acid sequence encoding the catalytic domain of H transferase and a nucleic acid sequence encoding localisation signal from Gal transferase. Still more preferably both nucleic acid sequences are derived from pigs. Even more preferably the nucleic acid encodes gTHT  
10 described herein.

The term "area of the cell" refers to a region, compartment or organelle of the cell. Preferably the area of the cell is a secretory organelle such as the Golgi apparatus.

15 In another aspect the invention provides an isolated nucleic acid molecule encoding a localisation signal of a glycosyltransferase. Preferably the signal encoded comprises an amino terminus of said molecule, more preferably it is the amino terminus of gal transferase. The  
20 gal transferase may be derived from or based on a gal transferase from any mammalian species, such as those described above. Particularly preferred sequences are those derived from pig, mouse or cattle.

The invention also extends to the proteins  
25 produced by the nucleic acid. Preferably the proteins are in an isolated form.

In another aspect the invention relates to a method of producing a nucleic acid encoding a chimeric enzyme, said enzyme comprising a catalytic domain of a  
30 first glycosyltransferase and a localisation signal of a second glycosyltransferase whereby when said nucleic acid is expressed in a cell said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with a second glycosyltransferase said method  
35 comprising operably linking a nucleic acid sequence encoding a catalytic domain from a first

glycosyltransferase to a nucleic acid sequence encoding a localisation signal of a second glycosyltransferase.

5 The term "operably linking" means that the nucleic acid sequences are ligated such that a functional protein is able to be transcribed and translated.

Those skilled in the art will be aware of the techniques for producing the nucleic acid. Standard techniques such as those described in Sambrook et al may be employed.

10 Preferably the nucleic acid sequences are the preferred sequences described above.

In another aspect the invention provides a method of reducing the level of a carbohydrate exhibited on the surface of a cell, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase whereby said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with said second glycosyltransferase and wherein said second glycosyltransferase is capable of producing said carbohydrate.

25 The term "reducing the level of a carbohydrate" refers to lowering, minimising, or in some cases, ablating the amount of carbohydrate displayed on the surface of the cell. Preferably said carbohydrate is capable of stimulating recognition of the cell as "non-self" by the immune system of an animal. The reduction of such a carbohydrate therefor renders the cell, or an organ composed of said cells, more acceptable to the immune system of an animal in a transplant situation or gene therapy situation.

35 The term "causing a nucleic acid to be expressed" means that the nucleic acid is introduced into the cell (i.e. by transformation/transfection or other suitable

means) and contains appropriate signals to allow expression in the cells.

The cell may be any suitable cell, preferably mammalian such as that of a New World monkey, ungulate (pig, sheep, goat, cow, horse, deer, camel, etc.) or other species such as dogs.

The invention also extends to cells produced by the above method and organs comprising the cells.

In another aspect the invention provides a method of producing a cell from one species which is immunologically acceptable to another species comprising reducing levels of carbohydrate on said cell which cause it to be recognised as non-self by the other species, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase whereby said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with said second glycosyltransferase and wherein said second glycosyltransferase is capable of producing said carbohydrate.

The term "immunologically acceptable" refers to producing a cell, or an organ made up of numbers of the cell, which does not cause the same degree of immunological reaction in the other species as a native cell from the one species. Thus the cell may cause a lessened immunological reaction, only requiring low levels of immunosuppression therapy to maintain such a transplanted organ or no immunosuppression therapy.

The cell may be from any of the species mentioned above. Preferably the cell is from a New World primate or a pig. More preferably the cell is from a pig.

The invention also extends to non-human transgenic animals harbouring the nucleic acid of the invention.

In another aspect the invention provides a retroviral packaging or producer cell which expresses the nucleic acid of the invention resulting in a cell which is immunologically acceptable to an animal having reduced levels of a carbohydrate on its surface, which carbohydrate is recognised as non-self by said species.

Preferably the species is a human, ape or Old World monkey.

The retroviral packaging cells or retroviral producer cells may be cells of any animal origin where it is desired to reduce the level of carbohydrates on its surface to make it more immunologically acceptable to a host. Such cells may be derived from mammals such as canine species, rodent or ruminant species and the like.

The retroviral packaging and/or producer cells may be used in applications such as gene therapy. General methods involving use of such cells is described in PCT/US95/07554 and the references discussed therein.

The invention also extends to a method of producing a retroviral packaging cell or a retroviral producer cell having reduced levels of a carbohydrate on its surface wherein the carbohydrate is recognised as non-self by a species comprising transforming/transfecting a retroviral packaging cell or a retroviral producer cell with the nucleic acid of the invention under conditions such that the chimeric enzyme is produced.

#### Brief Description of the Drawings

##### FIGURE LEGENDS

Figure 1 Schematic diagram of normal and chimeric glycosyltransferases

The diagram shows normal glycosyltransferases porcine  $\alpha(1,3)$ galactosyltransferase (GT) and human  $\alpha(1,2)$ fucosyltransferase (HT), and chimeric transferases ht-GT in which the cytoplasmic domain of GT has been completely replaced by the cytoplasmic domain of HT, and



gt-HT in which the cytoplasmic domain of HT has been entirely replaced by the cytoplasmic domain of GT. The protein domains depicted are cytoplasmic domain CYTO, transmembrane domain TM, stem region STEM, catalytic domain CATALYTIC. The numbers refer to the amino acid sequence of the corresponding normal transferase.

**Figure 2** Cell surface staining of COS cells transfected with normal and chimeric transferases. Cells were transfected with normal GT or HT or with chimeric transferases gt-HT or ht-GT and 48h later were stained with FITC-labelled lectin IB4 or UEA1. Positive-staining cells were visualised and counted by fluorescence microscopy. Results are from at least three replicates and values are +/- SEM.

**Figure 3.** RNA analysis of transfected COS cells. Northern blots were performed on total RNA prepared from COS cells transfected: Mock, mock-transfected; GT, transfected with wild-type GT; GT1-6/HT, transfected with chimeric transferase gt-HT; GT1-6/HT + HT1-8/GT, co-transfected with both chimeric transferases gt-HT and ht-GT; HT1-8/GT, transfected with chimeric transferase ht-GT; HT, transfected with normal HT; GT + HT, co-transfected with both normal transferases GT and HT. Blots were probed with a cDNA encoding GT (Top panel), HT (Middle panel) or  $\gamma$ -actin (Bottom panel).

**Figure 4.** Enzyme kinetics of normal and chimeric glycosyltransferases. Lineweaver-Burk plots for  $\alpha(1,3)$ galactosyltransferase ( $\square$ ) and  $\alpha(1,2)$ fucosyltransferase ( $\blacksquare$ ) to determine the apparent Km values for N-acetyl lactosamine. Experiments were performed in triplicate, plots shown are of mean values of enzyme activity of wild-type transferases, GT and HT, and chimeric proteins ht-GT and gt-HT in transfected COS cell

extracts using phenyl-B-D Gal and N-acetyl lactosamine as acceptor substrates.

**Figure 5. Staining of cells co-transfected with chimeric transferases**

- 5 Cells were co-transfected with cDNAs encoding normal transferases GT + HT (panels A, B), with chimeric transferases gt-HT + ht-GT (panels C, D), with HT + ht-GT (panels E, F) or with GT + gt-HT (panels G, H) and 48h later were stained with FITC-labelled lectin IB4 (panels A, C, E, G) or URAI (panels B, D, F, H).
- 10

Figure 6 is a representation of the nucleic acid sequence and corresponding amino acid sequence of pig secretor.

Figure 7 is a representation of the nucleic acid sequence and corresponding amino acid sequence of pig H.

15 Description of the Preferred Embodiment

- The nucleic acid sequences encoding the catalytic domain of a glycosyltransferase may be any nucleic acid sequence such as those described in PCT/US95/07554 provided that it encodes a functional catalytic domain with the desired glycosyltransferase activity.
- 20

Preferred catalytic domains from glycosyltransferase include H transferase and secretor. Preferably these are based on human or porcine sequences.

- The nucleic acid sequences encoding the localisation signal of a second transglycosylase may be any nucleic acid sequence encoding a signal sequence such as signal sequences disclosed in P A Gleeson, R D Teasdale & J Bourke, Targeting of proteins to the Golgi apparatus. Glycoconjugate J. (1994) 11: 381-394. Preferably the localisation signal is specific for the Golgi apparatus, more preferably for that of the trans Golgi. Still more preferably the localisation signal is based on that of Gal
- 25
- 30

transferase. Even more preferably the localisation signal is based on porcine, murine or bovine sequences. Even more preferably the nucleic acid encodes a signal sequence with following amino acid sequence (in single letter code):

5 MNVKGK (porcine), MNVKGK (mouse) or MVVKGK (bovine).

Vectors for expression of the chimeric enzyme may be any suitable vector including those disclosed in PCT/US95/07554 which is herein incorporated by reference.

10 The nucleic acid of the invention can be used to produce cells and organs with the desired glycosylation pattern by standard techniques such as those disclosed in PCT/US95/07554. For example, embryos may be transfected by standard techniques such as microinjection of the nucleic acid in a linear form into embryo (22). The embryos are  
15 then used to produce live animals, the organs of which may be subsequently used as donor organs for implantation.

Cells, tissues and organs suitable for use in the invention will generally be mammalian cells. Examples of suitable cells and tissues such as endothelial cells,  
20 hepatic cells, pancreatic cells and the like are provided in PCT/US95/07554.

The invention will now be described with reference to the following non-limiting Example.

#### ABBREVIATIONS

25 The abbreviations used are bp, base pair(s); FITC, fluorescein isothiocyanate; GT, galactosyltransferase; H substance,  $\alpha(1,2)$ fucosyl lactosamine; HT,  $\alpha(1,2)$ fucosyltransferase; PCR, polymerase chain reaction;

Example 1      Cytoplasmic domains of glycosyltransferases  
play a central role in the temporal action  
of enzymes

EXPERIMENTAL PROCEDURES

5                    **Plasmids** - The plasmids used were prepared using  
standard techniques (7); pGT encodes the cDNA for the  
porcine  $\alpha(1,3)$ galactosyltransferase (23), pHT encodes the  
cDNA for the  $\alpha(1,2)$ fucosyltransferase (human) (25).  
Chimeric glycosyltransferase cDNAs were generated by  
10 polymerase chain reaction as follows: an 1105 bp product  
ht-GT was generated using primers corresponding to the 5'  
end of ht-GT (5'-GCGGATCCATGTGGCTCCGGAGCC  
ATCGTCAGGTGGTTCTGTCAATGC TGCTTG-3') coding for nucleotides  
1-24 of HT (25) followed immediately by nucleotides 68-89  
15 of GT (8) and containing a BamHI site (underlined) and a  
primer corresponding to the 3' end of ht-GT (5'-  
GCTCTAGAGCGTCAGATGTTATT TCTAACCAATTATAC-3') containing  
complementarity to nucleotides 1102-1127 of GT with an XbaI  
site downstream of the translational stop site  
20 (underlined); an 1110 bp product gt-HT was generated using  
primers corresponding to the 5' end of gt-HT (5'-  
GCGGATCCATGAATGTCAAAGGAAGACTCTGCCTGGCCT TCCTGC-3') coding  
for nucleotides 49-67 of GT followed immediately by  
nucleotides 25-43 of HT and containing a BamHI site  
25 (underlined) and a primer corresponding to the 3' end of  
gt-HT (5'-GCTCTAGAGCGCTCAAGGCTTAG CCAATGTCCAGAG-3')  
containing complementarity to nucleotides 1075-1099 of HT  
with a XbaI site downstream of the translational stop site  
(underlined). PCR products were restricted BamHI/XbaI,  
30 gel-purified and ligated into a BamHI/XbaI digested pcDNA1  
expression vector (Invitrogen) and resulted in two plasmids  
pht-GT (encoding the chimeric glycosyltransferase ht-GT)  
and pgt-HT (encoding the chimeric glycosyltransferase gt-  
HT) which were characterised by restriction mapping,  
35 Southern blotting and DNA sequencing .

**Transfection and Serology** - COS cells were maintained in Dulbecco's modified Eagles Medium (DMEM) (Trace Biosciences Pty. Ltd. , Castle Hill, NSW, Australia) and were transfected (1-10 µg DNA/5 x 10<sup>5</sup> cells) using DEAE-Dextran (26); 48h later cells were examined for cell surface expression of H substance or Galα(1,3)Gal using FITC-conjugated lectins: IB4 lectin isolated from *Griffonia simplicifolia* (Sigma, St. Louis, MO) detects Galα(1,3)Gal (27); URAI lectin isolated from *Ulex europaeus* (Sigma, St. Louis, MO) detects H substance (28). H substance was also detected by indirect immunofluorescence using a monoclonal antibody (mAb) specific for the H substance (ASH-1952) developed at the Austin Research Institute, using FITC-conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) to detect mAb binding. Fluorescence was detected by microscopy.

**RNA Analyses** - Cytoplasmic RNA was prepared from transfected COS cells using RNazol (Biotecx Laboratories, Houston, TX), and total RNA was electrophoresed in a 1% agarose gel containing formaldehyde, the gel blotted onto a nylon membrane and probed with random primed GT or HT cDNA.

**Glycosyltransferase assays** - Forty-eight hours after transfection, cells were washed twice with phosphate buffered saline and lysed in 1% Triton X-100/ 100 mM cacodylate pH 6. 5/ 25 mM MnCl<sub>2</sub>, at 4°C for 30 min; lysates were centrifuged and the supernatant collected and stored at -70°C. Protein concentration was determined by the Bradford method using bovine serum albumin as standard (29). Assays for HT activity (30) were performed in 25 µl containing 3µM GDP-<sup>14</sup>C]fucose (specific activity 287 mCi/mmol, Amersham International), 5mM ATP, 50mM MOPS pH 6. 5, 20 mM MnCl<sub>2</sub>, using 2-10 µl of cell extract (approximately 15-20µg of protein) and a range of concentrations (7. 5 -75 mM) of the acceptor phenyl-B-D-galactoside (Sigma). Samples were incubated for 2h at 37°C and reactions terminated by the addition of ethanol and

water. The amount of  $^{14}\text{C}$ -fucose incorporated was counted after separation from unincorporated label using Sep-Pak C18 cartridges (Waters-Millipore, Millford, MA). GT assays (31) were performed in a volume of 25  $\mu\text{l}$  using 3  $\mu\text{M}$  UDP[ $^3\text{H}$ ]-Gal (specific activity 189  $\text{mCi}/\mu\text{mol}$ , Amersham International), 5  $\text{mM}$  ATP, 100  $\text{mM}$  cacodylate pH 6.5, 20  $\text{mM}$   $\text{MnCl}_2$ , and various concentrations (1 -10  $\text{mM}$ ) of the acceptor N-acetyl lactosamine (Sigma). Samples were incubated for 2h at 37°C and the reactions terminated by the addition of ethanol and water.  $^3\text{H}$ -Gal incorporation was counted after separation from non-incorporated UDP[ $^3\text{H}$ ]-Gal using Dowex 1 anion exchange columns (BDH Ltd., Poole, UK) or Sep-Pak Accell plus QMA anion exchange cartridges (Waters-Millipore, Millford, MA). All assays were performed in duplicate and additional reactions were performed in the absence of added acceptor molecules, to allow for the calculation of specific incorporation of radioactivity.

#### RESULTS

##### Expression of chimeric $\alpha(1,3)$ galactosyltransferase and $\alpha(1,2)$ fucosyltransferase cDNAs

We had previously shown that when cDNAs encoding  $\alpha(1,3)$ galactosyltransferase (GT) and  $\alpha(1,2)$ fucosyltransferase (HT) were transfected separately they could both function efficiently leading to expression of the appropriate carbohydrates: Gal $\alpha(1,3)$ Gal for GT and H substance for HT (32). However when the cDNAs for GT and HT were transfected together, the HT appeared to "dominate" over the GT in that H substance expression was normal, but Gal $\alpha(1,3)$ Gal was reduced. We excluded trivial reasons for this effect and considered that the localisation of the enzymes may be the reason. Thus, if the HT localisation signal placed the enzyme in an earlier temporal compartment than GT, it would have "first use" of the N-acetyl lactosamine substrate. However, such a "first use" if it occurred, was not sufficient to adequately reduce GT. Two

chimeric glycosyltransferases were constructed using PCR wherein the cytoplasmic tails of GT and HT were switched. The two chimeras constructed are shown in Fig.1: ht-GT which consisted of the NH<sub>2</sub> terminal cytoplasmic tail of HT attached to the transmembrane, stem and catalytic domains of GT; and gt-HT which consisted of the NH<sub>2</sub> terminal cytoplasmic tail of GT attached to the transmembrane, stem and catalytic domains of HT. The chimeric cDNAs were subcloned into the eukaryotic expression vector pcDNA1 and used in transfection experiments.

The chimeric cDNAs encoding ht-GT and gt-HT were initially evaluated for their ability to induce glycosyltransferase expression in COS cells, as measured by the surface expression of the appropriate sugar using lectins. Forty-eight hours after transfection COS cells were tested by immunofluorescence for their expression of Gal $\alpha$ (1,3)Gal or H substance (Table 1 & Fig. 2). The staining with IB4 (lectin specific for Gal $\alpha$ (1,3)Gal) in cells expressing the chimera ht-GT (30% of cells stained positive) was indistinguishable from that of the normal GT staining (30%) (Table 1 & Fig. 2). Similarly the intense cell surface fluorescence seen with UEA1 staining (the lectin specific for H substance) in cells expressing gt-HT (50%) was similar to that seen in cells expressing wild-type pHT (50%) (Table 1 & Fig. 2). Furthermore, similar levels of mRNA expression of the glycosyltransferases GT and HT and chimeric glycosyltransferases ht-GT and gt-HT were seen in Northern blots of total RNA isolated from transfected cells (Fig. 3). Thus both chimeric glycosyltransferases are efficiently expressed in COS cells and are functional indeed there was no detectable difference between the chimeric and normal glycosyltransferases.

**Glycosyltransferase activity in cells transfected with chimeric cDNAs encoding ht-GT and gt-HT**

To determine whether switching the cytoplasmic tails of GT and HT altered the kinetics of enzyme function, we compared the enzymatic activity of the chimeric glycosyltransferases with those of the normal enzymes in COS cells after transfection of the relevant cDNAs. By making extracts from transfected COS cells and performing GT or HT enzyme assays we found that N-acetyl lactosamine was galactosylated by both GT and the chimeric enzyme ht-GT (Fig 4. panel A) over a the 1-5mM range of substrate concentrations. Lineweaver-Burk plots showed that both GT and ht-GT have a similar apparent Michealis-Menten constant of Km 2. 6mM for N-acetyl lactosamine (Fig. 4. panel B). Further HT, and the chimeric enzyme gt-HT were both able to fucosylate phenyl-B-D-galactoside over a range of concentrations (7. 5 - 25 mM) (Fig. 4 panel C) with a similar Km of 2. 3mM (Fig. 4 panel D), in agreement with the reported Km of 2. 4mM for HT (25). Therefore the chimeric glycosyltransferases ht-GT and gt-HT are able to utilise N-acetyl lactosamine (ht-GT) and phenyl-B-D-galactoside (gt-HT) in the same way as the normal glycosyltransferases, thus switching the cytoplasmic domains of GT and HT does not alter the function of these glycosyltransferases and if indeed the cytoplasmic tail is the localisation signal then both enzymes function as well with the GT signal as with the HT signal.

**Switching cytoplasmic domains of GT and HT results in a reversal of the "dominance" of the glycosyltransferases**

The cDNAs encoding the chimeric transferases or normal transferases were simultaneously co-transfected into COS cells and after 48h the cells were stained with either IB4 or UEA1 lectin to detect Gal $\alpha$ (1,3)Gal and H substance respectively on the cell surface (Table 1 & Fig. 5). COS cells co-transfected with cDNAs for ht-GT + gt-HT (Fig 5



panel C) showed 30 % cells staining positive with IB4 (Table 1) but no staining on cells co-transfected with cDNAs for GT + HT (3%) (Fig. 5 panel A). Furthermore staining for H substance on the surface of ht-GT + gt-HT co-transfectants gave very few cells staining positive (5%) (Fig 5 panel D) compared to the staining seen in cells co-transfected with cDNAs for the normal transferases GT + HT (50%) (Fig. 5 panel B), ie. the expression of Gal $\alpha$ (1,3)Gal now dominates over that of H. Clearly, switching the cytoplasmic tails of GT and HT led to a complete reversal in the glycosylation pattern seen with the normal transferases i.e. the cytoplasmic tail sequences dictate the pattern of carbohydrate expression observed.

That exchanging the cytoplasmic tails of GT and HT reverses the dominance of the carbohydrate epitopes points to the glycosyltransferases being relocalized within the Golgi. To address this question, experiments were performed with cDNAs encoding glycosyltransferases with the same cytoplasmic tail: COS cells transfected with cDNAs encoding HT + ht-GT stained strongly with both UEA1 (50%) and IB4 (30%) (Table 1 & Fig. 5 panels E, F), the difference in staining reflecting differences in transfection efficiency of the cDNAs. Similarly cells transfected with cDNAs encoding GT + gt-HT also stained positive with UEA1 (50%) and IB4 (30%) (Table 1 & Fig. 5 panel G, H). Thus, glycosyltransferases with the same cytoplasmic tail leads to equal cell surface expression of the carbohydrate epitopes, with no "dominance" of one glycosyltransferase over the other observed, and presumably the glycosyltransferases localised at the same site appear to compete equally for the substrate.

In COS cells the levels of transcription of the cDNAs of chimeric and normal glycosyltransferases were essentially the same (Fig.3) and the immunofluorescence pattern of COS cells expressing the chimeric glycosyltransferases ht-GT and gt-HT showed the typical

staining pattern of the cell surface Gal $\alpha$ (1,3)Gal and H substance respectively (Table 1 & Fig. 2), the pattern being indistinguishable from that of COS cells expressing normal GT and HT. Our studies showed that the  $K_m$  of ht-GT for N-acetyl lactosamine was identical to the  $K_m$  of GT for this substrate, similarly the  $K_m$  of gt-HT for phenyl $\beta$ Dgalactoside was approximately the same as the  $K_m$  of HT for phenyl $\beta$ Dgalactoside (Fig. 3). These findings indicate that the chimeric enzymes are functioning in a cytoplasmic tail-independent manner, such that the catalytic domains are entirely functional, and are in agreement with those of Henion et al (23), who showed that an NH<sub>2</sub> terminal truncated marmoset GT (including truncation of the cytoplasmic and transmembrane domains) maintained catalytic activity and confirmed that GT activity is indeed independent of the cytoplasmic domain sequence.

If the Golgi localisation signal for GT and HT is contained entirely within the cytoplasmic domains of the enzymes, then switching the cytoplasmic tails between the two transferases should allow a reversal of the order of glycosylation. Co-transfection of COS cells with cDNA encoding the chimeric glycosyltransferases ht-GT and gt-HT caused a reversal of staining observed with the wild type glycosyltransferases (Fig. 5), demonstrating that the order of glycosylation has been altered by exchanging the cytoplasmic tails. Furthermore, co-transfection with cDNA encoding glycosyltransferases with the same cytoplasmic tails (i. e. HT + ht-GT and GT + gt-HT) gave rise to equal expression of both Gal $\alpha$ (1,3)Gal and H substance (Fig. 5). The results imply that the cytoplasmic tails of GT and HT are sufficient for the localisation and retention of these two enzymes within the Golgi.

To date only twenty or so of at least one hundred predicted glycosyltransferases have been cloned and few of these have been studied with respect to their Golgi localisation and retention signals (34). Studies using the

elongation transferase N-acetylglucosaminyltransferase I (33-37), the terminal transferases  $\alpha(2,6)$ sialyltransferase (24-26) and  $\beta(1,4)$ galactosyltransferase (38-40) point to residues contained within the cytoplasmic tail, transmembrane and flanking stem regions as being critical for Golgi localisation and retention. There are several examples of localisation signals existing within cytoplasmic tail domains of proteins including the KDEL and KKXX motifs in proteins resident within the endoplasmic reticulum (41,42) the latter motif also having been identified in the cis Golgi resident protein ERGIC-53 (43) and a di-leucine containing peptide motif in the mannose-6-phosphate receptor which directs the receptor from the trans-Golgi network to endosomes (44). These motifs are not present within the cytoplasmic tail sequences of HT or GT or in any other reported glycosyltransferase. To date a localisation signal in Golgi resident glycosyltransferases has not been identified and while there is consensus that transmembrane domains are important in Golgi localisation, it is apparent that this domain is not essential for the localisation of all glycosyltransferases, as shown by the study of Munro (45) where replacement of the transmembrane domain of  $\alpha(2,6)$ sialyltransferase in a hybrid protein with a poly-leucine tract resulted in normal Golgi retention. Dahdal and Colley (46) also showed that sequences in the transmembrane domain were not essential to Golgi retention. This study is the first to identify sequence requirements for the localisation of  $\alpha(1,2)$ fucosyltransferase and  $\alpha(1,3)$ galactosyltransferase within the Golgi. It is anticipated that other glycosyltransferases will have similar localisation mechanisms.

Example 2      Use of secretor in construction of a  
chimeric enzyme

Construct is made using PCR and subcloning as described in Example 1 such that amino acids #1 to #6 of the pig  $\alpha$ 1,3galactosyltransferase (MNVKGR) replace amino acids #1 to 5 of the pig secretor (Fig 6). Constructs are tested as described in Example 1.

Example 3      Use of pig H transferase in construction of  
a chimeric enzyme

Construct is made using PCR and subcloning as described in Example 1 such that amino acids #1 to #6 of the pig  $\alpha$ 1,3galactosyltransferase (MNVKGR) replace amino acids #1 to 8 of the pig H transferase (Fig 7). Constructs are tested as described in Example 1.

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THE AUSTIN RESEARCH INSTITUTE

By Its Patent Attorneys:

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Attorneys of Australia

TABLE 1

EXPRESSION OF GAL $\alpha$ (1,3)GAL AND H SUBSTANCE BY COS CELLS  
TRANSFECTED WITH cDNAs ENCODING NORMAL AND CHIMERIC  
GLYCOSYLTRANSFERASES

5	COS cells transfected with cDNA encoding:	%IB4 positive cells	%UEAI positive cells
	GT	30	0
	HT	0	50
	ht-GT	30	0
10	gt-HT	3	50
	GT+HT	3	50
	ht-GT+gt-HT	33	5
	GT+gt-HT	30	30
	GT+ht-GT	30	0
15	HT+ht-GT	30	30
	HT+gt-HT	0	50
	Mock	0	0

20 Transfected COS cells were stained with FITC-labelled IB4  
(lectin specific for Gal $\alpha$ (1,3)Gal or UEAI (lectin specific  
for H substance) and positive staining cells were  
visualized and counted by fluorescence microscopy. Results  
are from at least three replicates.

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FIGURE 1

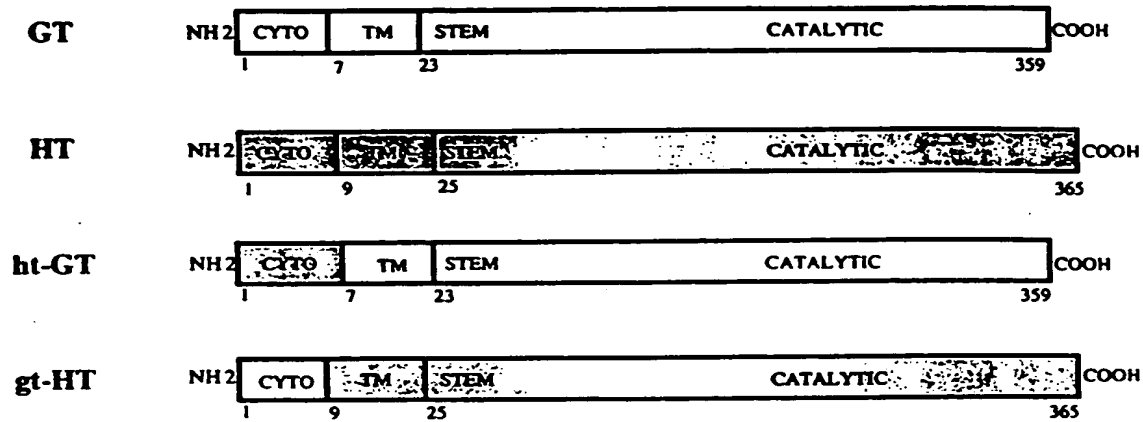
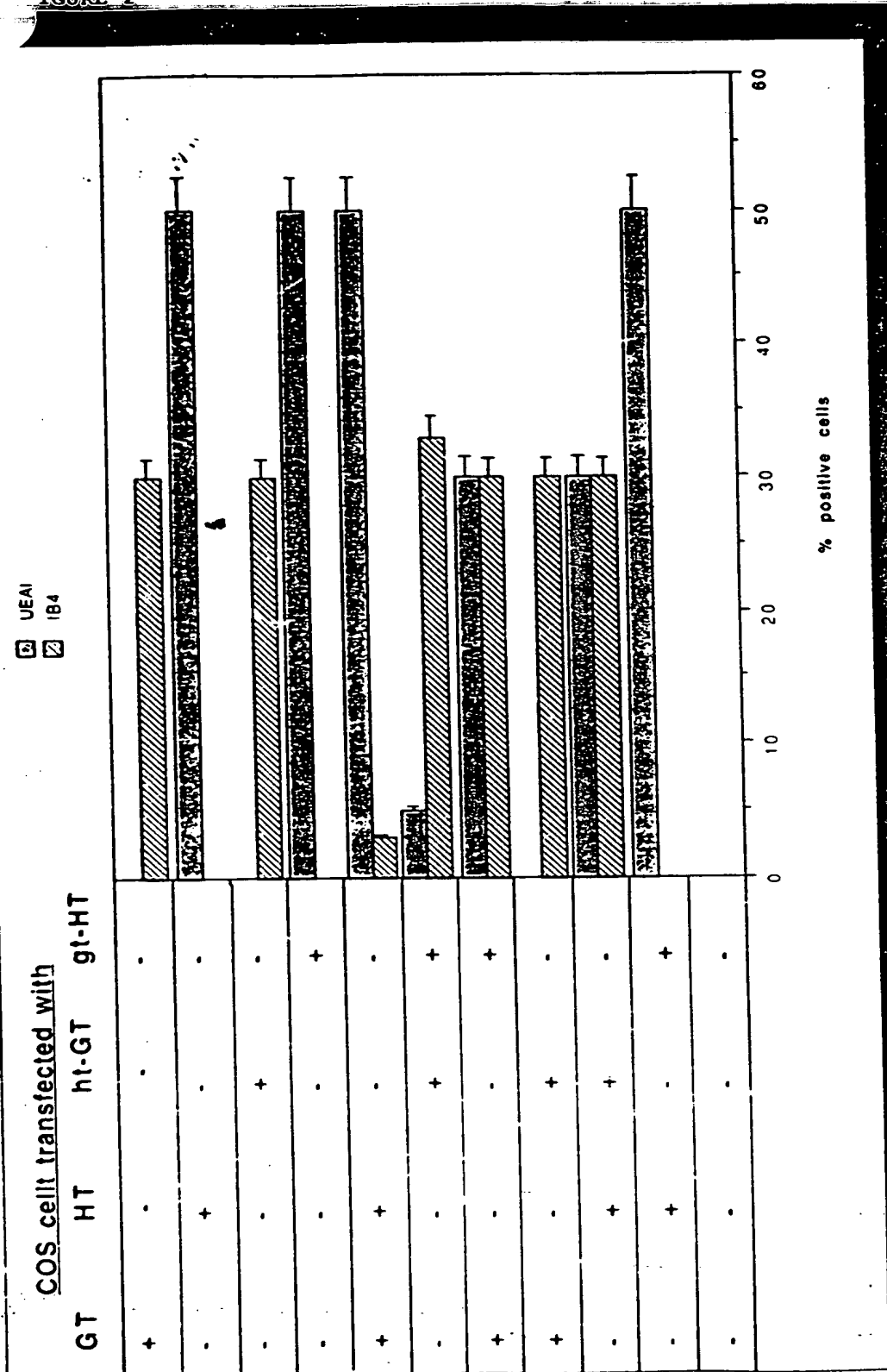


FIGURE 2



Western blot analysis showing HT and  $\gamma$ -actin expression. The lanes are labeled: Mock, GT, GT1-6/HT, GT1-6/HT + HT1-8/GT, HT1-8/GT, HT, and GT + HT1. The top row shows HT expression, with bands present in GT, GT1-6/HT, GT1-6/HT + HT1-8/GT, HT1-8/GT, and GT + HT1 lanes. The middle row shows HT expression, with bands present in GT1-6/HT, GT1-6/HT + HT1-8/GT, HT1-8/GT, and HT lanes. The bottom row shows  $\gamma$ -actin expression, with bands present in all lanes, serving as a loading control.

FIGURE 4

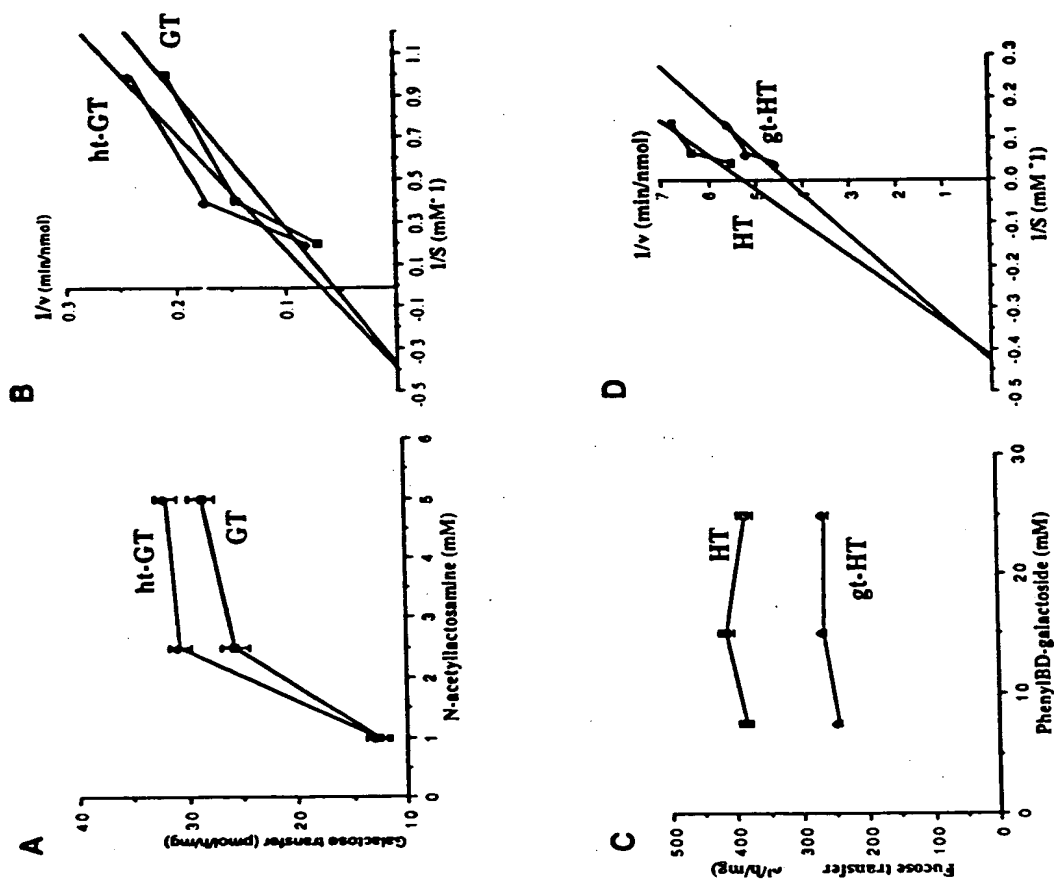




FIGURE 6

## PORCINE SECRETOR SEQUENCE

CT ACA GCC ATG CTC AGC ATG CAG GCA TCC TTC TTC TTC CCC AGG GGT CCC TTC ATC CTC	17 59
F V F T A S T I F H L Q Q R H V E I Q P	37 119
TTT GTG TTC AGG GCT TCC ACC ATA TTT CAC CTT CAG CAG AGG ATG GTG AAG ATT CAA CCC	
T W E L Q H V T Q V T T E S P S S P Q L	57 179
ACC TGG GAG TTA CAG ATG GTG ACC CAG GTG ACC ACA GAG AGC CCC TGG AGC CTC CAG CTG	
K Q H M T I M A I G R L G H Q M G E Y A	77 239
AAG GGC ATG TGG AGG ATC AAT GGC ATC GGC CCG CTG GCG AAC CAG ATG GCG GAG TAC GGC	
T L Y A L A R H N G R P A F I P P E H H	97 293
ACC CTG TAC GCG CTG GCG AGG ATG AAC GCG CCG CCG GGC TTC ATC CCG LAC GAG ATG CAC	
S T L A P I F R I T L F V L H A S T A R	117 359
ACC AGG CTG GCG CCC ATC TTC AGG ATC ACC CTC CCG GTC CTG CAC GCG ACC AGC GCG CCG	
R I P W Q N Y H L N D W H E E R Y R H I	137 419
AGG ATC CCC TGG CAG AAC TAC CAC CTG AAC GAC TGG ATG GAG GAG CCG TAC CCG CAC ATC	
P G E Y V R L T G Y P C S W T F Y K H L	157 479
CCG GCG GAG TAC GTG CCG CTC AGG GCG TAC CTC TGC TCC TGG ACC TTC TAC CAC CAC CTG	
R T E I L R E F T L H N H V R E E A Q D	177 539
CGC ACC GAG ATC CTC CCG GAG TTC ACC CTG CAT AAC CAC CTG CCG GAG GCG CCG CAG GAT	
F L R G L R V N G S R P S T Y V G V H V	157 599
TTC CTG CCG GCT CTG CCG GTG AAC GCG AGC CGA CCG AGT ACC TAC GTG GCG GTG CAC GTG	
R R G D Y V H V M P N V W K G V V A D R	217 659
CGC CCG GAG GAC TAC GTG CAC GTG ATG CCC AAC GTG TGG AAG GCG GTG GTG GCG GAC CCG	
R Y L E Q A L D W F R A R Y R S P V P V	237 719
CGG TAC CTG GAG CAG GCG CTG GAC TGG TTC CCG GCT CCG TAC CCG TCC CCG GTC TTT GTG	
V S S N G H A W C R E N I N A S R G D V	257 779
GTG TCC AGC AAC GCG ATG GCG TGG TGT CCG GAA AAC ATC AAT GCG TCG CCG GCG GAT GTG	
V F A G H G I E C S P A K D F A L L T Q	277 839
GTG TTT GCG GCG AAT GCG ATC GAG GCG TCC CCC GCG AAA GAC TTC GCG CTG CTC AGG CAG	
C N H T V M T I G T F G I W A Y L A G	297 899
TGT AAC CAC ACT GTG ATG ACC ATT GCG AGC TTC GCG ATC TGG GCG TAC CTT GCT GGT	
G E T I Y L A N Y T L P D S P F L K L F	317 959
GGA GAG ACC ATC TAC CTG GCG AAT TAC AGC CCG GAG TCT CCG TTC CTC AAA CTC TTT	
K P E A A F L P E W I G I E A D L S P L	337 1019
AAG CCC GAG GCA GCG TTC CTG CCC GAG TGG ATT GCG ATC GAG GCA GAC CTG TCC CCA CTC	
L K I	340 1043
CTT AAG CAC TGA TGT GCG CTG TCC	



**FIGURE 7**

## FIG 8 TRANSFERASE

CT	GCA	GCC	AGC	TGG	GTC	CCC	AGC	CCC	CCC	CAC	CTC	TGT	CTG	ACC	TTC	CTG	CTA	GTC	TGT	17
V	L	A	A	I	F	F	L	N	V	Y	Q	D	L	F	Y	S	G	L	D	37
GTT	TTA	GCA	GCA	ATT	TTC	TTC	CTG	AAC	GTC	TAT	GAA	GAC	CTC	TTT	TAC	AGT	GCC	TTA	GAC	119
L	L	A	L	C	P	D	H	N	V	V	S	S	P	V	A	I	F	C	L	57
CTG	CTG	GCC	CTG	TGT	CCA	GAC	CAT	AAC	GTC	GTA	TCA	TCT	CCC	GTC	GCC	ATA	TTC	TGC	CTG	179
A	G	T	P	V	N	P	N	A	S	D	S	C	P	K	H	P	A	S	F	77
GCC	GCC	AGC	CCG	GTA	CAC	CCC	AAC	GCC	TGC	GAT	TCC	TGT	CCC	AAG	CAT	CCT	GCC	TCC	TTT	239
S	G	T	W	T	I	Y	P	D	G	R	F	G	N	Q	M	G	Q	Y	A	97
TCC	GCG	ACC	TGG	ACT	ATT	TAC	CCG	GAT	GCC	CCG	TTT	GCG	AAC	CAG	ATG	GGA	CAG	TAT	GCC	299
T	L	L	A	L	A	Q	L	N	G	R	Q	A	F	I	Q	P	A	M	H	117
AGC	CTG	CTG	GCC	CTG	GCG	CAG	CTC	AAC	GCC	CCC	CAG	GCC	TTC	ATC	CAG	CCT	GAC	ATG	CAC	359
A	V	L	A	P	V	F	R	I	T	L	P	V	L	A	P	E	V	D	R	137
GCC	GTC	CTG	GCC	CCC	GTC	TTC	CCC	ATC	ACG	CTG	CCT	GTC	CTG	GCG	CCC	GAG	GTA	GAC	AGC	419
H	A	P	W	R	E	L	H	D	W	M	S	E	D	Y	A	H	L			157
CAC	GCT	CCT	TGG	CCG	GAG	CTG	GAG	CTT	CAC	GAC	TGG	ATG	TCC	GAG	GAT	TAT	GCC	CAC	TTA	479
K	E	P	W	L	R	L	T	G	F	P	C	S	W	T	F	F	H	H	L	177
ANG	GAG	CCC	TGG	CTG	ANG	CTC	ACC	GCC	TTC	CCC	TGC	TCC	TGG	ACC	TTC	TTC	CAC	CAC	CTC	539
R	E	Q	I	R	S	E	F	T	L	H	D	H	L	R	Q	E	A	Q	G	197
CCG	GAG	CAG	ATC	CCG	AGC	GAG	TTC	ACC	CTG	CAC	GAC	CAC	CTT	CCG	CAA	GAG	GCC	CAG	GGG	599
V	L	S	O	F	R	L	P	R	T	G	D	R	P	S	T	F	V	G	V	217
GTA	CTG	AGT	CAG	TTC	CCT	CTA	CCC	CCC	ACA	GGG	GAC	CCC	CCC	AGC	ACC	TTC	GTG	GGG	GTC	659
B	V	R	R	G	D	Y	L	R	V	M	P	K	R	W	K	G	V	V	G	237
CAC	GTC	CCC	CCG	GGG	GAC	TAT	CTG	CCT	GTG	ATG	CCC	AAG	CCG	TGG	AAG	GGG	GTG	GTG	GCT	719
D	G	A	Y	L	Q	Q	A	M	D	W	F	R	A	R	Y	E	A	P	V	257
GAC	GCC	GCT	TAC	CTC	CAG	CAG	GCT	ATG	GAC	TGG	TTT	CCG	GCC	GGA	TAC	GAA	GCC	CCC	GTC	779
F	V	V	T	S	N	G	M	E	W	C	R	K	N	I	D	T	S	R	G	277
TTT	GTG	GTC	ACC	AGC	AAC	GCC	ATG	GAG	TGG	TCC	CCG	AAG	AAC	ATC	GAC	ACC	TCC	CCG	GGG	839
D	V	I	F	A	G	D	G	R	E	A	A	P	A	R	D	F	A	L	L	297
GAC	GTG	ATC	TTT	GCT	GCC	GAT	GGC	CCG	GAG	GCC	GCG	CCC	GCC	AGG	GAC	TTT	GGG	CTG	CTG	899
V	Q	C	N	H	T	I	M	T	I	G	T	F	G	F	W	A	A	Y	L	317
GTG	CAG	TGC	AAC	CAC	ACC	ATC	ATG	ACC	ATT	GGC	ACC	TTC	GCC	TTC	TGG	GCC	GCC	TAC	CTG	959
A	G	G	D	T	I	Y	L	A	N	K	T	L	P	T	S	S	F	L	K	337

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